Compensation Theory

Compensation control requirements for the proper instrument setup:

- **Unstained control** sample should be the **same origin** and prepared with the **same protocol** as the cells used in the experiment
- **Single-color controls:**
  - Need to have single-color controls for **all** the fluorochromes used in the experiment
  - All controls should be treated in the same way as samples (such as: incubation, fixation, permeabilization)
  - Should contain particles (either cells or compensation beads) stained with **each fluorescence marker individually;**

  - ***Note: Most common fluorescence markers:*** *fluorochrome-labeled antibody (directly conjugated or via biotin-SA-fluorochrome system), fluorescent proteins, functional probes, etc.*
  - Within each single-color control tube the **unstained and positive particles** have to be of the **same origin** (either cells or beads);
  - **Level of fluorescence** in the controls should be **same or brighter** than in the samples.

Compensation goals — unstained and stained particles should show the **same mean value (or median** for digital instruments) **in all the "bystander" channels except the one "dedicated"** for it.

Bead Compensation Controls

Reason to use BD FACSComp Bead as Single Stain Controls:

- **Cell** numbers are **limiting**
- Antigen is **dimly** expressed
- Antibody has **low affinity** to receptor
- **Positive** population is **rare**
- Use **tandem** dyes

FACSComp Bead — Kits e.g. from BD, Biolegend, eBiosciences

Details:

- Each Kit contains **two vials** with bead polystyrene particles;
  - **Beads uncoated** that have no binding capacity; used for negative population
  - **Beads coated** with Goat **anti-Ig kappa,** which bind light chain-bearing immunoglobulin; used for positive population. Be sure to use same Fluorochrome-conjugated monoclonal antibodies as actually used for cell staining in your experiment
- **Note:** there are various FACSComp Bead Kits available with the coated beads of different specificity (Hamster, Rat or Mouse)

BD FACSComp Beads labeling procedure (implemented and recommended by FCF-Berg):

1 **Verify origin** of your antibody (Hamster, Rat or Mouse) to specify which beads should be used
2 **Mix Beads** well by shaking the bottle
3 **Pre-dilute** anti-Ig Beads using 400 µl of PBS for 1 drop of Beads
4 **Dispense** 100 µl of pre-diluted Beads per single color control sample
5 **Add** 5 µl of pre-diluted **primary Ab** to the Beads
6 **Incubate** 20 min at RT, mix occasionally
7 If applicable, add 5 µl of pre-diluted secondary Ab with Fluorochrome
8 **Incubate** 20 min at RT, mix occasionally
9 **Add** 50 µl of pre-diluted **uncoated Beads** to each sample
10 **Make** separate tube with **uncoated** beads for **Negative Control**

Use immediately for Multi-color compensation **or store** at +4°C for up to 2 weeks